CLINICAL STUDY

Expression of the *CD11c* gene in subcutaneous adipose tissue is associated with cytokine level and insulin resistance in women with polycystic ovary syndrome

Tao Tao^{1,*}, Shengxian Li^{1,*}, Aimin Zhao², Yanyun Zhang³ and Wei Liu¹

¹Division of Endocrinology and Metabolism, Department of Internal Medicine and ²Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, Shanghai Jiaotong University School of Medicine, Renji Hospital, 1630 Dongfang Road, Pudong, Shanghai 200127, People's Republic of China and ³Joint Immunology Laboratory of Institute of Health Sciences and Shanghai Institute of Immunology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China

(Correspondence should be addressed to W Liu; Email: sue_liuwei@163.com; Y Zhang; Email: yyzhang@sibs.ac.cn)

*(T Tao and S Li contributed equally to this work)

Abstract

Objective: Alterations in the phenotypes of macrophages in adipose tissue play a key role in inflammation and insulin resistance (IR). The phenotypes of macrophages in subcutaneous adipose tissue (SAT) and the relationship between proinflammation markers and IR in women with polycystic ovary syndrome (PCOS) remain unclear. The objectives of this study are to characterize the gene expression of macrophage markers and cytokines in the SAT of PCOS women and to estimate their relationships with circulating levels of cytokines and IR.

Methods: The cross-sectional study involves 16 PCOS women and 18 normal control women. Cytokines and macrophage markers in the circulation and SAT were determined using ELISA, quantitative PCR, or immunofluorescence staining. IR was estimated using the homeostasis model assessment (HOMA-IR).

Results: The gene expression levels of *CD11c* along with *TNF α* and leptin in SAT remained significantly higher in PCOS women than in normal women ($P < 0.05$). However, no significant differences were found in *CD68* mRNA expression in SAT between women with and without PCOS ($P > 0.05$). Furthermore, *CD11c* mRNA abundance provided a stronger contribution to models predicting serum levels of *TNF α* (sTNF α) than did *CD68* mRNA abundance. Lastly, increased sTNF α was associated with increased HOMA-IR in PCOS women, and this association was independent of both overall and visceral adiposity.

Conclusion: The high expression level of *CD11c* mRNA in SAT was proved to be an important feature in PCOS women. Furthermore, *CD11c* mRNA abundance made a stronger contribution to models predicting sTNF α in which existing proinflammatory properties might significantly contribute to the pathogenesis of IR in PCOS women.

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Introduction

Polycystic ovary syndrome (PCOS) is known to affect 6–10% of reproductive-aged women (1). Insulin resistance (IR) has been considered to be the most important etiological aspect of reproductive and metabolic abnormalities in PCOS, in part due to the high prevalence of obesity in this disorder (2, 3). A chronic, low-grade inflammation occurring in adipose tissue is at least in part responsible for obesity-induced IR (4). Recent evidence of subclinical inflammation is present in women with PCOS (5, 6) and might play an important role in the pathogenesis of PCOS (7). However, the precise role of dominant cell-extrinsic mechanisms, such as alterations in the levels of circulating adipokines and the occurrence of metabolic

tissue inflammation that modulate peripheral insulin action in women with PCOS, has not been established.

In recent years, the relationship between the inflammation status in adipose tissue and IR has attracted wide attention. Findings from recent studies indicate that adipose tissue inflammation and the related metabolic dysregulation are connected to the presence of several immune cell types that were infiltrating into the adipose tissue. These include not only macrophages but also lymphocytes and other cells (8, 9). Among the immune cells that infiltrate adipose tissue in obesity, macrophages are functionally and numerically dominant (10, 11). However, findings of Arkan *et al.* (12) showed that lack of the proinflammatory enzyme inhibitor of nuclear factor κ -B kinase subunit β (IKK β) in macrophages does not

develop in diet-induced IR. Interestingly, recent studies (10, 11) have revealed that classically activated (M1) macrophages, which release cytokines such as IL1 β , IL6, and TNF α creating a proinflammatory environment that blocks adipocyte insulin action, have a crucial role in contributing to the pathogenesis of diet-induced IR. Furthermore, temporal analysis of adipose tissue in obesity has revealed that the recruitment of M1 macrophages coincides with the appearance of necrotic adipocytes and with the onset of IR (13).

CD11c is a member of the β 2 integrins, which has been consistently reported to be unregulated in macrophages infiltrating white adipose tissue (14). Further, CD11c+ macrophages are seen to encircle necrotic adipocytes (15, 16), thereby forming crown-like structures (13) and remodeling adipose tissue structures. Recently, a study showed that CD11c promoter-dependent conditional deletion of adipose tissue macrophages (ATMs) improves insulin sensitivity, which indicates that CD11c+ subsets of human ATMs are much more important in pathophysiology of IR than previously thought (17). Furthermore, the findings of Wu *et al.* (18) suggest that CD11c deficiency in diet-induced obese mice does not alter weight gain but decreases inflammation and ameliorates IR and glucose intolerance. It is suggested that the conditions of CD11c expression may deter obesity progression by inducing inflammation and IR in AT. CD11c+ macrophages play a key role in obesity-induced IR and obesity is a common phenotype in women with PCOS. Therefore, an important consideration is whether CD11c+ macrophages, acting as a characteristic of proinflammatory subset of classically activated macrophages, are also implicated in the pathogenesis of IR in women with PCOS.

Despite the importance of CD11c expression in diet-induced obesity as a regular aspect of IR and inflammation, much less is known about CD11c expression in subcutaneous adipose tissue (SAT) and their systemic effect on IR in women with PCOS. Additionally, the mechanisms for adipose dysfunction and subclinical inflammation in the pathophysiology of IR in women with PCOS remain unclear. Thus, we performed this study to test the characterization of adipose tissue dysfunction and proinflammatory markers in the circulation and in SAT of women with PCOS in relation to IR.

Materials and methods

A total of 34 subjects were included in this study. They were recruited from the outpatient Department of Endocrinology and Gynecology of Shanghai Renji Hospital between 2009 and 2010. Of these patients (age range 18–35 years), there were 16 women with PCOS (BMI range 23.8–29.64 kg/m²) and 18 control women (BMI range 25.23–28.19 kg/m²). All patients

with PCOS were recruited from among patients seeking care for oligomenorrhea and/or hirsutism at the outpatient wards of the participating clinics. The PCOS diagnosis was based on the National Institutes of Health 1990 criteria. These include: i) clinical evidence of hyperandrogenism and/or hyperandrogenemia; ii) oligo-ovulation; and iii) the exclusion of related disorders, including nonclassical 21-hydroxylase-deficient adrenal hyperplasia, hyperprolactinemia, thyroid dysfunction, Cushing's syndrome, or androgen-producing tumors (19). The control women with regular menstrual cycles and without a family history of endocrine abnormality or hirsutism were recruited through an advertisement in a local newspaper. Control subjects were enrolled into the study if they met the following eligibility criteria: i) a healthy state, as determined by medical history, physical examination, screening laboratory evaluations, and transvaginal ultrasound; ii) without known heredity for type 2 diabetes mellitus and hypertension; iii) absence of changes ($\geq 10\%$) in body weight during the last year preceding the screening; iv) normal exercise and drinking habits; and v) no current regular medication (such as oral contraceptives or other drugs known to alter inflammation status). All study evaluations and procedures were conducted in accordance with the guidelines of the Helsinki Declaration on human experimentation. The study was approved by the ethics committee of Shanghai Renji hospital and all subjects provided written informed consent.

Laboratory analysis

All laboratory evaluations were performed at 0800 h after an overnight fast during the early follicular phase (days 2–5) of a spontaneous menstrual cycle, except in subjects with amenorrhea >3 months who were examined randomly. Fasting glucose and insulin samples were stored at 4 °C and analyzed on the day of sampling. Plasma glucose was determined using the glucose oxidase methodology. All measurements were performed with Roche reagents (D 2400 and E170 Modular Analytics modules with Roche/Hitachi analyzers; Roche Diagnostics). Insulin levels were measured by RIA. The intra-assay coefficient of variation (CV) of insulin assays was 5.5 and <10% respectively. Insulin sensitivity was evaluated by the quantitative insulin sensitivity check index (QUICKI) method. $QUICKI = 1/(\log(I0) (\mu U/ml) + \log(G0) (mg/dl))$. The IR index (IRI) was calculated using the homeostatic assessment model of IR (HOMA-IR). $HOMA-IR = (FINS \times FPG)/22.5$ (FINS, fasting serum insulin; FPG, fasting plasma glucose). Islet β cell function was evaluated by the homeostasis model assessment of β -cell function (HOMA- β), $HOMA-\beta = 20 \times FINS / (FPG - 3.5)$. Competitive electrochemiluminescence immunoassays on the Elecsys autoanalyzer 2010 (Roche Diagnostics) were used to quantify serum

total testosterone and estradiol levels. Sex hormone-binding globulin (SHBG) levels were measured by chemiluminescent immunoassay (Elecsys autoanalyzer 2010, Roche Diagnostics) and were validated for plasma SHBG (20). The CV for SHBG using this methodology was 6%. Free testosterone values were calculated based on total testosterone and SHBG levels according to the method outlined by Vermeulen *et al.* (21) assuming an albumin concentration of 4 g/dl (<http://www.issam.ch/freetesto.htm>). Serum leptin (s-leptin), adiponectin, and TNF α were measured using a commercial ELISA kit (R&D, Minneapolis, MN, USA). The intra-assay and interassay CV were below 6 and 10% respectively.

Adipose tissue collection

Adipose tissue samples (~500 mg) were obtained surgically from SAT through a small incision in the lower abdomen. Specimens were transported immediately to the laboratory in a HEPES salts buffer containing 4% BSA and 2 mM pyruvate (pH 7.4) and were finely minced. The tissue specimens were immediately frozen in liquid nitrogen and stored at -80°C until total RNA isolation was undertaken.

RNA isolation, RT, and real-time quantitative PCR

Total RNA was isolated from the SAT and cells using TRIzol reagent (Invitrogen) and was purified using the Qiagen RNeasy Mini Kit (Qiagen). Isolated RNA was reverse transcribed into cDNA by following the protocol from Superscript^{III} First-Strand Synthesis System (Invitrogen). The mRNA expression of leptin, adiponectin, TNF α , IL6, IL10, peroxisome proliferator-activated receptor γ (PPAR γ (PPARG)), CD11c, and CD68 was determined by real-time quantitative PCR (QPCR). The QPCRs were performed in triplicate using a LightCycler instrument (Roche Diagnostics) with SYBR Premix Ex Taq (TaKaRa, Dalian, Japan) and were normalized using β -actin. Assay gene expression primers used are indicated in Table 1. Oligonucleotide primers were designed using the Primer Express 2.0 software (ABI, New York, NY, USA). The primer was

synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Sangon, Shanghai, China) Amplification was performed in 10 μl reactions using an amount of 4 ng mRNA equivalent and a two-step standard protocol was employed following the manufacturer's instructions: amplification was performed during 40 cycles (pre-denaturation: 10 s, 95°C , one cycle; PCR: 5 s, 94°C ; 31 s, 60°C , 40 cycles; dissociation stage). All primers used were validated for PCR efficiency. For each sample, ΔCp values were determined (Cp of the target gene minus Cp of the β -actin gene). Fold changes in expression were calculated according to the transformation: fold increase = $2^{-\text{difference in } \Delta\text{Cp}}$.

Immunofluorescence staining

A small amount of tissue was fixed in 4% PBS-buffered paraformaldehyde for immunofluorescence. Frozen 20 μm sections cut from adipose tissue embedded in optimal cutting temperature compound (OCT) were fixed in acetone. Primary antibodies used were rabbit MABs to CD11c (Abcam, Cambridge, UK) for classically activated macrophages. For single-antibody stains, sections were treated with 10% goat serum (Boster, Wuhan, China) and then washed in PBS before overnight incubation with primary antibody. Slides were then labeled with Cy3-conjugated rabbit anti-rat IgG (Jackson Laboratories, East Brisbane, QLD, Australia). Then slides were mounted with Dapi mount media (Vector Labs, Inc., Burlingame, CA, USA) and examined under immunofluorescence microscopy (Nikon, Tokyo, Japan). Immunopositive CD11c+ expression cells were counted in a field of 0.04 mm^2 , and only those with nuclei observable by DAPI staining were counted.

Statistical analysis

Distributions of continuous variables were tested for normality by use of the Kolmogorov–Smirnov test. Portions of results not normally distributed, based on the normal quartile plot, were log-transformed for all statistical analyses and reported back-transformed in their original units. All results were reported as

Table 1 Primer sequences.

Gene	Forward (5'–3')	Reverse (5'–3')
Leptin	ATTTACACACGTCAGTCTCTC	TCACGTTTCTGGAAGGCATAC
Adiponectin	GAGATCCAGGTCTTATTGGTCC	CAATCCCACACTGAATGCTG
TNF α	GAGTGACAAGCCTGTAGCCC	GATGAGGTACAGGCCCTCTG
CD11c	TGACATTGCATCGAAGCCCTC	TCCGTACCCTCAATGGCAAAG
CD68	ATGGCGGTGGAGTACAATGTGT	AGAATGATGCTCGAGTTGCTGC
PPAR γ	CAGGCTTGCTGAACGTGAAG	GGAGCACCTTGGCGAACA
IL6	GGTACATCCTCGACGGCATCT	GT GCCTCTTTGCTGCTTTCAC
IL10	GGGAGAACCTGAAGACCCTCA	TGCTCTT GTTTTCACAGGGAAG
β -Actin	CCTTCTACAATGAGCTGCGTG	ACAGCCTGGATAGCAACGTAC

means, or geometric means for log-transformed variables, with 95% CIs. Comparisons between groups were made using Student's *t*-test. *P* values <0.05 were considered significant. All statistical analyses were performed using SPSS version 13.0 (Statistical Package for the Social Sciences).

With this assumption, the study had >85% power to detect differences in adipose tissue mRNA expression between these two groups, given an α -error of 0.05. Gene expression differences were made using Wilcoxon and Mann–Whitney *U* tests. The relationships between serum levels of cytokines and adipose tissue *CD11c* expression and IR were evaluated by Spearman's correlation tests. Stepwise multiple linear regression analysis was performed for selected data, with statistical significance defined as *P*<0.05. Two forward, stepwise, multiple linear regression analyses were performed. The first model was performed to determine the best model to predict IR as estimated by HOMA-IR considering biologically plausible predictors, i.e. age, BMI, WHR, adiponectin, leptin, and TNF α . These independent variables were selected based on the evaluation of the literature and because their correlations with insulin sensitivity were significant in the univariate analyses. Based on the results of the first model, a second model was created to determine the best model to predict serum levels of TNF α considering age, BMI, WHR, and *CD11c*, *CD11c/CD68* as plausible independent variables.

Results

Hormonal and biochemical features

The clinical characteristics and biochemical variables for the two groups of women according to PCOS status

are summarized in Table 2. As expected, there were no significant differences in age, BMI, waist circumstances, WHR, fasting glucose, or estradiol between women with and without PCOS. However, mean total and free testosterone values were also significantly higher in the PCOS group compared with the BMI-matched control group (*P*=0.003 and *P*=0.000 respectively). Mean SHBG values were significantly lower in the PCOS group compared with the BMI-matched control group (*P*=0.001). Meanwhile, fasting insulin and HOMA-IR values were significantly higher in women with PCOS than in the BMI-matched normal controls (*P*=0.003 and *P*=0.011 respectively). Mean HOMA-IS and QUICKI values were significantly lower in women with PCOS than in normal controls (*P*=0.002 and *P*=0.013 respectively).

Characterization of adipose tissue dysfunction in PCOS patients

To identify the features of adipose tissue dysfunction and subclinical inflammation state, we compared circulating level of adiponectin, leptin, and TNF α . As shown in Table 2, serum levels of leptin and TNF α remained significantly higher in women with PCOS than in normal control women (*P*=0.034 and *P*=0.000 respectively). However, there were no significant differences in serum levels of adiponectin between women with and without PCOS (*P*=0.157). Furthermore, we performed quantitative PCR to detect the relative amounts of mRNA for adiponectin, leptin, TNF α , and PPAR γ in SAT between women with and without PCOS. As shown in Fig. 1, the expression levels of leptin and TNF α mRNA in SAT were significantly higher in women with PCOS than those in BMI-matched controls (*P*=0.039 and *P*=0.042

Table 2 Clinical and biochemical variables in women with polycystic ovary syndrome (PCOS) and normal control women. Data are means (95% CI) unless noted.

Variable	Control	PCOS	<i>P</i> value
<i>n</i>	18	16	NA
Age (years)	25.4 (24.3–26.6)	24.6 (22.1–27.2)	0.517
BMI (kg/m ²)	26.71 (25.23–28.19)	26.72 (23.80–29.64)	0.992
Waist circumference (cm)	87.19 (82.87–91.52)	86.37 (80.06–98.1)	0.818
WHR	0.88 (0.85–0.91)	0.87 (0.84–0.90)	0.54
FBG (mmol/l)	5.31 (4.99–5.61)	4.96 (4.72–5.19)	0.074
Fasting insulin (mIU/l)	7.85 (6.09–9.60)	12.61 (9.90–15.32)	0.003
HOMA-IR	1.84 (1.42–2.28)	2.79 (2.17–3.40)	0.011
HOMA-IS	95.98 (70.37–121.59)	190.13 (133.12–247.15)	0.002
QUICKI	0.36 (0.34–0.37)	0.33 (0.32–0.35)	0.013
Estradiol (pmol/l)	295.17 (197.02–393.32)	225.07 (150.35–299.77)	0.238
Total testosterone (nmol/l)	1.63 (1.42–1.85)	2.96 (2.18–3.74)	0.003
SHBG (nmol/l)	62.71 (51.12–74.29)	36.23 (27.68–44.77)	0.001
Free testosterone* (pmol/l)	19.75 (16.44–23.06)	53.74 (38.85–68.62)	0.000
Adiponectin (mg/l)	9.47 (7.99–10.94)	7.88 (6.07–9.70)	0.157
Leptin (μ g/l)	41.08 (29.14–52.93)	61.97 (45.45–78.13)	0.034
TNF α (ng/l)	30.69 (22.41–38.98)	68.66 (49.52–87.80)	0.000

P value for unpaired *t*-test for difference between two groups. WHR, waist-to-hip ratio; QUICKI, quantitative insulin-sensitivity check index; HOMA-IR, homeostasis model assessment-insulin resistance index; FBG, fasting blood glucose; SHBG, sex hormone-binding globulin; to convert glucose to mg/dl, divide by 0.05551. *geometric means.

Figure 1 The gene expression of *CD11c* (A), *CD68* (B), *TNFα* (C), leptin (D), adiponectin (E), *PPARγ* (F), *IL6* (G), and *IL10* (H) in adipose tissue in women with and without PCOS. Data are mean $\pm$ S.E.M. *P* value for Wilcoxon and Mann–Whitney *U* tests for difference between two groups <0.05 for *CD11c*, *TNFα*, leptin, adiponectin, *PPARγ*, *IL6*, and *IL10*. * $P < 0.05$, ** $P < 0.01$.

respectively). In addition, we found that the mRNA expression levels of adiponectin and ratios of leptin to adiponectin were similar between PCOS and non-PCOS groups ($P = 0.53$ and $P = 0.077$ respectively). Lastly, the mRNA expression levels of *PPARγ* in SAT were significantly lower in women with PCOS than in BMI-matched controls ($P = 0.008$).

Macrophage infiltration into adipose tissue and phenotype of ATM subsets

In addition, we performed quantitative PCR to evaluate the mRNA expression levels of *CD68*, a macrophage-specific marker, and *CD11c* in SAT between women with PCOS and controls. As shown in Fig. 1, the expression levels of *CD11c* mRNA were significantly higher in women with PCOS than in BMI-matched controls ($P = 0.036$). However, the expression levels of *CD68* mRNA and ratios of *CD11c* to *CD68* mRNA were not

significantly different between women with PCOS and normal control women ($P = 0.677$ and $P = 0.962$ respectively). Meanwhile, no significant differences in the mRNA expression levels of *IL6* and *IL10* were found between PCOS women and control subjects ($P = 0.067$ and $P = 0.14$ respectively).

To identify macrophage phenotypes present in adipose tissue, we performed immunofluorescent staining with specific antibodies. As shown in Fig. 2, the presence of macrophages, identified by positive staining for *CD11c* in SAT, which is viewed as a marker of proinflammatory macrophages marker, clearly indicated the presence of specific subsets of macrophages in the SAT of women with PCOS.

Correlation between *CD11c* mRNA expression levels and IR-related gene/biochemical characteristics

As shown in Fig. 3, considering all women with PCOS, serum levels of both *TNFα* ($r = 0.824$, $P = 0.000$) and leptin ($r = 0.582$, $P = 0.018$) had positive linear relationships with the expression levels of *CD11c*

Figure 2 Immunofluorescence staining of *CD11c*-positive cells in SAT obtained from women with PCOS (A) and control women (B) (magnification, $\times 40$) is shown. The white arrow indicates *CD11c*-positive (red) cells in subcutaneous adipose tissue quantified by counting in a field of 0.04 mm^2 .

Figure 3 Relationship between *CD11c* gene expression in adipose tissue and serum TNF α (A) and s-leptin (B) in women with PCOS ($n=16$). P value for Spearman's correlation tests.

mRNA. In addition, as shown in Fig. 4, a strong positive linear correlation was observed between *CD11c* mRNA expression levels and IR estimated by HOMA-IR ($r=0.577$, $P=0.019$). However, neither *PPAR* γ mRNA expression ($r=-0.103$, $P=0.704$) nor other gene (*TNF* α , leptin, and adiponectin) expression levels correlated significantly with *CD11c* mRNA expression levels ($P>0.05$ for all). Finally, we do not find that *CD11c* and *CD68* mRNA expression levels have strong linear relationships with estradiol or with total and free testosterone values ($P>0.05$ for all).

Determinants of IR as estimated by HOMA-IR

To investigate the independent determinants of IR among PCOS women studied, we performed a forward, stepwise, multiple regression analysis with HOMA-IR as the dependent variable and *CD11c* mRNA, BMI, s-leptin, and sTNF α as potential contributors to IR. In the analysis, only sTNF α was selected as an independent variable for the final model of HOMA-IR. In the final model for HOMA-IR, the adjusted R^2 was

0.998 ($P<0.0001$) and the regression coefficients (β) were 0.001 ($P=0.003$) for sTNF α . Thus, sTNF α was an independent predictor of IR as estimated by HOMA-IR in women with PCOS.

Determinants of serum TNF α

As TNF α mainly secreted from classically activated macrophages, we hypothesized that *CD11c* mRNA abundance might be an important predictor of sTNF α level. After sTNF α was selected as a better predictor of HOMA-IR than BMI, *CD11c* mRNA, and s-leptin in the first linear regression analysis, a second model was created, again using forward, stepwise, linear regression analysis, with sTNF α as the dependent variable and *CD11c* mRNA, *CD68* mRNA, and s-leptin as potential independent variables. Considering these variables, only *CD11c* mRNA was selected as an independent variable for the final model predicting sTNF α . The adjusted R^2 for the model predicting sTNF α was 0.701 ($P=0.003$), and the regression coefficients (β) were 1.118 ($P=0.000$). Thus, the expression level of *CD11c* mRNA was an independent predictor of sTNF α level in women with PCOS.

Discussion

In diet-induced obesity, most of the infiltrating ATMs were polarized into the M1 phenotype and the presence of a high number of these *CD11c* $^{+}$ macrophages was associated with a local inflammation status and IR in adipose tissue (22). However, data concerning the link among *CD11c* $^{+}$ macrophages, system inflammation, and IR in women with PCOS are limited. The issue of whether the change in inflammation in adipose tissue poses a major defect for IR in women with PCOS was also unsettled. The main finding of this study is the

Figure 4 Relationship between *CD11c* gene expression in adipose tissue and insulin resistance estimated by HOMA-IR in women with PCOS ($n=16$). P value for Spearman's correlation tests.

demonstration that existing high expression levels of classically activated inflammatory macrophages in abdominal SAT, as estimated by *CD11c* expression, was an important feature found in women with PCOS. This kind of phenotypic switch of macrophage in adipose tissue is perhaps a more important indicator of IR than total macrophage infiltration as estimated by *CD68* mRNA in adipose tissue in women with PCOS. In addition, the increased *CD11c* mRNA expression level is seen to be related to increased sTNF α and s-leptin levels in PCOS women. Furthermore, sTNF α provided a stronger contribution to models predicting IR than did leptin or adiponectin in women with PCOS. Lastly, *CD11c* mRNA provided a stronger contribution to models predicting sTNF α level in women with PCOS.

It is noteworthy that chronic low-level inflammation, a key contributor to the pathogenesis of PCOS, was shown in recent studies (23, 24). However, previous studies that assessed the relationship between sub-clinical inflammation and IR in women with PCOS have yielded conflicting results (25, 26). Based on our present data, we find that, compared with BMI-matched controls, serum TNF α level is significantly increased in women with PCOS. Additionally, in a forward stepwise, linear regression analysis performed to determine predictors of IR as estimated by HOMA-IR in women with PCOS, sTNF α was shown to be an independent predictor of HOMA-IR after controlling for BMI and age. However, we found that neither s-leptin nor adiponectin were independent predictors of HOMA-IR after controlling for BMI in this study. Therefore, we speculated that TNF α may be more important than leptin and adiponectin in predicting IR in women with PCOS after controlling for BMI and may be involved in the development of PCOS. The observed association between TNF α and IR in our study is consistent with other studies suggesting that the TNF α has the most potent IR properties (27) in women with PCOS. Moreover, our results are in agreement with the previous studies showing that serum levels of inflammatory markers are increased in overweight patients with PCOS compared with weight-matched controls (28). However, in conflict with our results, Olszanecka-Glinianowicz *et al.* (29) reported that they failed to display any differences between overweight controls and overweight patients with PCOS in terms of circulating TNF α and IL6. In addition, our data show that the gene expression level of *TNF α* in SAT is obviously higher in women with PCOS than in BMI-matched control women. However, we do not find that the expression levels of *IL6* mRNA were significantly different between women with PCOS and normal control women. Findings from this study seem to contrast with the reports from the laboratories of Lindholm *et al.* (30). They showed that overweight rather than the PCOS diagnosis *per se* appears to be the main explanatory variable for elevated adipose tissue inflammation in patients with PCOS. Several

explanations for the apparent disparity in these findings can be hypothesized, including differences in study subject demographics (specifically ethnicity, different PCOS criteria) and clinical characteristics (i.e. higher prevalence of obesity in this study). In our study, we used the 1990 NIH PCOS criteria, but the studies by Lindholm *et al.* (30) based their diagnosis of PCOS on the Rotterdam diagnostic criteria, and thus represent a more heterogenous group of women. Lastly, the correlation analyses do not show sTNF α correlated with the expression of *TNF α* mRNA in SAT in this study. This may suggest that alterations in sTNF α in women with PCOS contribute to the phenotypic presentation of the syndrome via additional mechanisms dependent on posttranscriptional regulation.

As TNF α mainly secreted from ATMs exists in close correlation with IR in women with PCOS, we speculate that macrophage infiltration into adipose tissue should act as a feature of paramount importance, leading to adipose-derived inflammation and IR in women with PCOS. We do not find that the expression levels of *CD68*, *IL6*, and *IL10* mRNA in SAT have a significant difference between PCOS women and BMI-matched control women though. However, we find that increased *CD11c* mRNA abundance was associated with sTNF α ($P=0.000$) and HOMA-IR ($P=0.019$) in women with PCOS. More importantly, immunofluorescence staining of *CD11c* clearly indicates the presence of specific subsets of macrophages in the SAT in women with PCOS in this study. Hence, our results suggested that alterations in phenotype of ATMs may contribute to the system inflammation and IR in women with PCOS. However, the precise mechanism of macrophage activation remains unknown. Our data suggest that the PCOS diagnosis *per se* appears to be the main explanatory variable for elevated adipose tissue inflammation in women with PCOS. These findings seem to support the reports from the laboratories of González *et al.* They show that hyperandrogenism may be a potential mechanism for inflammation in PCOS by activating mononuclear cells (MNC) in the fasting state and increasing MNC sensitivity to glucose (31). Unfortunately, we do not find that *CD11c* and *CD68* mRNA expression levels have strong linear relationships with serum estradiol or with total and free testosterone values. Interestingly, we find that the expression of enzymes synthesizing testosterone and enzymes inactivating DHT and progesterone are higher in SAT of PCOS patients compared with BMI-matched controls in our previous study (32). Moreover, correlation analysis indicated that increased leptin expression may be negatively related to local DHT level. This suggests that a complex network among local sex hormone and adipocytokines exists in SAT in women with PCOS. Furthermore, Alemany (33) suggested that the differences in androgen availability and handling may help to explain some of the sex-related differences in the onset and development of the metabolic syndrome.

Hence, a possible explanation may lie in the role of the local sex hormones in macrophage phenotype switch through changing the microenvironment of adipocytes, which increases local immune response and inflammation in PCOS women. However, given the cross-sectional design of our study, causality cannot be established. The precise mechanisms of macrophage activation in SAT in women with PCOS must be further investigated.

Several limitations of our study should be considered. The study includes a limited number of participants, which is the reason we cannot separate subtle groups according to BMI, but we instead analyze the data after adjusting for BMI. Another limitation is that only adipose tissue biopsies from one anatomical location were available for this study. Recently, genomic and proteomic profiling studies of morbidly obese PCOS omental adipose tissue have revealed changes in components of several biological pathways (34, 35). Hence, there might be differences in the inflammatory status of visceral adipose tissue in women with PCOS (36). However, it should be emphasized that the subcutaneous fat in the truncal region appears to contribute more to both the IR and the circulating NEFA pool than do adipose tissue depots elsewhere in the body (37), and it is not likely that the visceral tissue would directly account for the increased circulating concentrations of plasma cytokines and chemokines (38, 39). Finally, CD11c+ is not the only marker for staining M1-macrophages known in humans. However, CD11c+ macrophage acting as an important characteristic of pro-inflammation was associated with a local inflammation status in diet-induced obesity. On the other hand, we found decreased expression levels of *PPAR γ* mRNA in SAT in women with PCOS in this study, which gene can stimulate M2 polarization of ATMs and change systemic insulin sensitivity (40).

In conclusion, we find an important link between macrophage activation status and IR in PCOS women in this study. The remodeling of SAT might be proposed to be active participants in the development of IR in women with PCOS. Further investigation is warranted to determine both the mechanisms by which inflamed adipose tissue is altered in PCOS and the potential contributions of alterations in the proinflammatory cytokines to ovulatory dysfunction in PCOS women.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- 1 Diamanti-Kandarakis E, Kouli CR, Bergiele AT, Filandra FA, Tsianateli TC, Spina GG, Zapanti ED & Bartzis MI. A survey of the polycystic ovary syndrome in the Greek island of Lesbos: hormonal and metabolic profile. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 4006–4011. (doi:10.1210/jc.84.11.4006)
- 2 Dunaif A. Insulin resistance and the polycystic ovary syndrome: mechanism and implication for pathogenesis. *Endocrine Reviews* 1997 **18** 774–800. (doi:10.1210/er.18.6.774)
- 3 Hahn S, Tan S, Sack S, Kimmig R, Quadbeck B, Mann K & Janssen OE. Prevalence of the metabolic syndrome in German women with polycystic ovary syndrome. *Experimental and Clinical Endocrinology & Diabetes* 2007 **115** 130–135. (doi:10.1055/s-2007-967093)
- 4 Gregor MF & Hotamisligil GS. Inflammatory mechanisms in obesity. *Annual Review of Immunology* 2011 **29** 415–445. (doi:10.1146/annurev-immunol-031210-101322)
- 5 Escobar-Morreale HF, Luque-Ramírez M & González E. Circulating inflammatory markers in polycystic ovary syndrome: a systematic review and metaanalysis. *Fertility and Sterility* 2011 **95** 1048–1058.e1–2. (doi:10.1016/j.fertnstert.2010.11.036)
- 6 Samy N, Hashim M, Sayed M & Said M. Clinical significance of inflammatory markers in polycystic ovary syndrome: their relationship to insulin resistance and body mass index. *Disease Markers* 2009 **26** 163–170. (doi:10.3233/DMA-2009-0627)
- 7 Repaci A, Gambineri A & Pasquali R. The role of low-grade inflammation in the polycystic ovary syndrome. *Molecular and Cellular Endocrinology* 2011 **335** 30–41. (doi:10.1016/j.mce.2010.08.002)
- 8 Lolmède K, Duffaut C, Zakaroff-Girard A & Bouloumié A. Immune cells in adipose tissue: key players in metabolic disorders. *Diabetes & Metabolism* 2011 **37** 283–290. (doi:10.1016/j.diabet.2011.03.002)
- 9 Lumeng CN & Saltiel AR. Inflammatory links between obesity and metabolic disease. *Journal of Clinical Investigation* 2011 **121** 2111–2117. (doi:10.1172/JCI57132)
- 10 Ferrante AW Jr. Obesity-induced inflammation: a metabolic dialogue in the language of inflammation. *Journal of Internal Medicine* 2007 **262** 408–414. (doi:10.1111/j.1365-2796.2007.01852.x)
- 11 Odegaard JI & Chawla A. Alternative macrophage activation and metabolism. *Annual Review of Pathology* 2011 **6** 275–297. (doi:10.1146/annurev-pathol-011110-130138)
- 12 Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J & Karin M. IKK- β links inflammation to obesity-induced insulin resistance. *Nature Medicine* 2005 **11** 191–198. (doi:10.1038/nm1185)
- 13 Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW II, DeFuria J, Jick Z, Greenberg AS & Obin MS. Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes* 2007 **56** 2910–2918. (doi:10.2337/db07-0767)
- 14 Wu H, Gower RM, Wang H, Perrard XY, Ma R, Bullard DC, Burns AR, Paul A, Smith CW, Simon SI & Ballantyne CM. Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. *Circulation* 2009 **119** 2708–2717. (doi:10.1161/CIRCULATIONAHA.108.823740)
- 15 Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS & Obin MS. Adipocyte death

- defines macrophage localization and function in adipose tissue of obese mice and humans. *Journal of Lipid Research* 2005 **46** 2347–2355. (doi:10.1194/jlr.M500294-JLR200)
- 16 Lumeng CN, Bodzin JL & Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *Journal of Clinical Investigation* 2007 **117** 175–184. (doi:10.1172/JCI29881)
 - 17 Patsouris D, Li PP, Thapar D, Chapman J, Olefsky JM & Neels JG. Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. *Cell Metabolism* 2008 **8** 301–309. (doi:10.1016/j.cmet.2008.08.015)
 - 18 Wu H, Perrard XD, Wang Q, Perrard JL, Polsani VR, Jones PH, Smith CW & Ballantyne CM. CD11c expression in adipose tissue and blood and its role in diet-induced obesity. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2010 **30** 186–192. (doi:10.1161/ATVBAHA.109.198044)
 - 19 Zawadzki JK & Dunaif A. Diagnostic criteria for polycysticovary syndrome: towards a rational approach. In *Current Issues in Endocrinology and Metabolism: Polycystic Ovary Syndrome*, pp 377–384. Eds A Dunaif, J Givens, F Haseltine & G Merriam, New York: Blackwell, 1992.
 - 20 Reynders M, Anckaert E, Schiettecatte J & Smits J. Evaluation of a new automated electrochemiluminescent sex hormone-binding globulin (SHBG) immunoassay. *Clinical Chemistry and Laboratory Medicine* 2005 **43** 86–89. (doi:10.1515/CCLM.2005.013)
 - 21 Vermeulen A, Verdonck L & Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 3666–3672. (doi:10.1210/jc.84.10.3666)
 - 22 Chawla A, Nguyen KD & Goh YP. Macrophage-mediated inflammation in metabolic disease. *Nature Reviews. Immunology* 2011 **11** 738–749. (doi:10.1038/nri3071)
 - 23 González F. Inflammation in polycystic ovary syndrome: underpinning of insulin resistance and ovarian dysfunction. *Steroids* 2012 **77** 300–305. (doi:10.1016/j.steroids.2011.12.003)
 - 24 González F, Rote NS, Minium J & Kirwan JP. Increased activation of nuclear factor kappaB triggers inflammation and insulin resistance in polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 1508–1512. (doi:10.1210/jc.2005-2327)
 - 25 Kelly CCJ, Lyall H, Petrie JR, Gould GW, Connell JMC & Sattar N. Low grade chronic inflammation in women with polycystic ovarian syndrome. *Journal of Clinical Endocrinology and Metabolism* 2001 **86** 2453–2455. (doi:10.1210/jc.86.6.2453)
 - 26 Mohlig M, Spranger J, Osterhoff M, Ristow M, Pfeiffer AF, Schill T, Schlösser HW, Brabant G & Schöfl C. The polycystic ovary syndrome per se is not associated with increased chronic inflammation. *European Journal of Endocrinology* 2004 **150** 525–532. (doi:10.1530/eje.0.1500525)
 - 27 Hotamisligil GS, Shargill NS & Spiegelman BM. Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* 1993 **259** 87–91. (doi:10.1126/science.7678183)
 - 28 Gonzalez F, Rote NS, Minium J & Kirwan JP. Evidence of proatherogenic inflammation in polycystic ovary syndrome. *Metabolism* 2009 **58** 954–962. (doi:10.1016/j.metabol.2009.02.022)
 - 29 Olszanecka-Glinianowicz M, Banas M, Zahorska-Markiewicz B, Janowska J, Kocelak P, Madej P & Klimek K. Is the polycystic ovary syndrome associated with chronic inflammation per se? *European Journal of Obstetrics & Gynecology and Reproductive Biology* 2007 **133** 197–202. (doi:10.1016/j.ejogrb.2006.10.037)
 - 30 Lindholm A, Blomquist C, Bixo M, Dahlbom I, Hansson T, Sundström Poromaa I & Burén J. No difference in markers of adipose tissue inflammation between overweight women with polycystic ovary syndrome and weight-matched controls. *Human Reproduction* 2011 **26** 1478–1485. (doi:10.1093/humrep/der096)
 - 31 González F, Nair KS, Daniels JK, Basal E & Schimke JM. Hyperandrogenism sensitizes mononuclear cells to promote glucose-induced inflammation in lean reproductive-age women. *American Journal of Physiology. Endocrinology and Metabolism* 2012 **302** E297–E306. (doi:10.1152/ajpendo.00416.2011)
 - 32 Wang L, Li S, Zhao A, Tao T, Mao X, Zhang P & Liu W. The expression of sex steroid synthesis and inactivation enzymes in subcutaneous adipose tissue of PCOS patients. *Journal of Steroid Biochemistry and Molecular Biology* 2012 **132** 120–126. (doi:10.1016/j.jsbmb.2012.02.003)
 - 33 Alemany M. Do the interactions between glucocorticoids and sex hormones regulate the development of the metabolic syndrome? *Frontiers in Endocrinology* 2012 **3** 27. (doi:10.3389/fendo.2012.00027)
 - 34 Corton M, Botella-Carretero JL, Benguria A, Villuendas G, Zaballos A & San Millan JL. Escobar-Morreale HF, Peral B. Differential gene expression profile in omental adipose tissue in women with polycystic ovary syndrome. *Journal of Clinical Endocrinology and Metabolism* 2007 **92** 328–337. (doi:10.1210/jc.2006-1665)
 - 35 Corton M, Botella-Carretero JL, Lopez JA, Camafeita E, San Millan JL, Escobar-Morreale HF & Peral B. Proteomic analysis of human omental adipose tissue in the polycystic ovary syndrome using two-dimensional difference gel electrophoresis and mass spectrometry. *Human Reproduction* 2008 **23** 651–661. (doi:10.1093/humrep/dem380)
 - 36 Carmina E, Chu MC, Moran C, Tortoriello D, Vardhana P, Tena G, Preciado R & Lobo R. Subcutaneous and omental fat expression of adiponectin and leptin in women with polycystic ovary syndrome. *Fertility and Sterility* 2008 **89** 642–648. (doi:10.1016/j.fertnstert.2007.03.085)
 - 37 Garg A. Regional adiposity and insulin resistance. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 4206–4210. (doi:10.1210/jc.2004-0631)
 - 38 Murdolo G, Hammarstedt A, Sandqvist M, Schmelz M, Herder C, Smith U & Jansson PA. Monocyte chemoattractant protein-1 in subcutaneous abdominal adipose tissue: characterization of interstitial concentration and regulation of gene expression by insulin. *Journal of Clinical Endocrinology and Metabolism* 2007 **92** 2688–2695. (doi:10.1210/jc.2006-2814)
 - 39 Despres JP & Lemieux I. Abdominal obesity and metabolic syndrome. *Nature* 2006 **444** 881–887. (doi:10.1038/nature05488)
 - 40 Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L, Red Eagle A, Vats D, Brombacher F, Ferrante AW & Chawla A. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* 2007 **447** 1116–1120. (doi:10.1038/nature05894)

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